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Flap loop of GluD2 binds to Cbln1 and induces presynaptic differentiation

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Abstract

Glutamate receptor $\delta 2$ (GluD2) is selectively expressed on the postsynaptic spines at parallel-fiber (PF)-Purkinje neuron (PN) synapses. GluD2 knockout mice show a reduced number of PF-PN synapses, suggesting that GluD2 is involved in synapse formation. Recent studies revealed that GluD2 induces presynaptic differentiation in a manner dependent on its N-terminal domain (NTD) through binding of Cbln1 secreted from cerebellar granule neurons. However, the underlying mechanism of the specific binding of the NTD to Cbln1 remains elusive. Here, we have identified the flap loop (Arg321-Trp339) in the NTD of GluD2 (GluD2-NTD) as a crucial region for the binding to Cbln1 and the induction of presynaptic differentiation. Both induction of presynaptic differentiation and binding of Cbln1 were abolished in the HEK cells expressing not wild-type GluD2 but GluD2 with mutations in the flap loop. Especially, single amino acid substitution of either Arg321 or Trp323 to alanine was sufficient to disable the GluD2 function. Finally, a homology model of GluD2-NTD suggested that the flap loop is located at the distal end, which appears consistent with an interaction with Cbln1 and a presynaptic varicosity.

Keywords

Glutamate receptor $\delta 2$, N-terminal domain, flap loop, Cbln1, synapse formation

Abbreviations

iGluR, ionotropic glutamate receptor; NTD, N-terminal domain; PF, parallel fiber; PN, Purkinje neuron

Introduction

Ionotropic glutamate receptors (iGluRs) are classified into 4 families: AMPA-type, NMDA-type, kainate-type and δ -type according to their agonists and sequence homology. AMPA, NMDA, and kainate receptors function as cation channels gated by glutamate and mediate fast excitatory synaptic transmission. On the other hand, δ receptors, consisting of glutamate receptor $\delta 1$ and $\delta 2$ (GluD1 and GluD2), are unique in the following two respects: they do not bind to glutamate [1-4], and they function as synaptic adhesion molecules. GluD2 plays essential roles in normal development and synaptic transmission in the cerebellum [5]. GluD2 is localized on the postsynaptic spines of parallel-fiber (PF) –Purkinje-neuron (PN) synapses [6]. GluD2 knockout mice show impairment of cerebellar long-term depression, multiple innervation of climbing fibers to PNs and a reduced number of PF-PN synapses [7, 8]. Previously, we showed that GluD2 plays a crucial role in synapse formation. HEK cells expressing GluD2 induced presynaptic differentiation through the extracellular N-terminal domain (NTD) [9].

Cbln1, a member of the C1q necrosis factor superfamily, is expressed in and secreted from cerebellar granule neurons [10]. Cbln1 forms a trimer via its C-terminal C1q domain, and one Cbln1 trimer binds to another via N-terminal disulfide bonds [11]. Cbln1 knockout mice show similar phenotypes to GluD2 knockout mice [12], such as a reduced number of PF-PN synapses. Recent works showed that Cbln1 binds to both postsynaptic GluD2-NTD and presynaptic β -neurexin, and suggested that their triad interaction is involved in the formation of PF-PN synapses [13, 14].

All iGluRs are homo- or hetero-tetramers, and each monomer has three transmembrane helices (M1, M3 and M4) and one short helix forming a channel pore (M2). The extracellularly located N-terminal sequences are divided into the N-terminal domain (NTD) and S1, which forms the ligand binding domain (LBD) together with S2 located between M3 and M4 [15]. NTD of iGluR consists of ~400 amino acids and is required for selective heterotetramer formation among iGluR subtypes [16, 17]. It is homologous to bacterial leucine/isoleucine/valine binding protein and to the glutamate binding domain of metabotropic glutamate receptors [18, 19]. Here, we have addressed the structural basis of the synaptogenic activity of GluD2-NTD.

Material and methods

Cell Cultures

Cerebella were dissected out from newborn mouse pups and incubated in Ca^{2+} - and Mg^{2+} -free HBSS containing 0.1 % trypsin and 0.05 % DNase for 15 min at 37 °C [20]. Neurons were dissociated by trituration and seeded on poly-D-lysine-coated coverslips in DMEM/F12-based medium containing 2% fetal bovine serum. The next day, 75% of the medium was replaced with serum-free medium. Thereafter, half of the medium was replaced with serum-free medium on 4 and 10 days after dissociation. To inhibit glial proliferation, cytosine β -D-arabinofuranoside (5 μM) was added to the medium from 4 days after dissociation.

Expression Vectors

The expression vector of HA- GluD2 was constructed as previously described [20]. GluD2 and all mutants were tagged with an HA-epitope sequence at their N-terminal ends. Sequences of HA-GluD2 with mutated residues in the flap loop were generated by PCR and inserted into pCAGplay. The expression vector of myc-Cbln1 was constructed as follows. Myc tag-encoding sequence was inserted into pCAGplay at the 3'-end of the signal peptide region (pCAGplay-myc). The Cbln1 sequence was cloned from a cDNA library prepared from mouse cerebella and inserted into the pCAGplay-myc at the 3'-end of myc tag region.

Homology modeling of GluD2-NTD domain

To construct a homology model of δ 2-NTD, the sequence of mouse GluD2-NTD was submitted to the EsyPred3D web server (www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred/) [21] as a query using rat GluA2-NTD (PDB ID: 3H5V_A) for a template. Comparing the calculated structure of GluD2-NTD with the template structure of GluA2-NTD by Swiss-PdbViewer 4.0 (www.expasy.org/spdbv/) gave root-mean-square values of 1.22 Å using the C α atoms of 356 residues.

Coculture assay

The coculture assay was performed as previously described [9]. Briefly, HEK cells were transfected with EGFP (pEGFP-N1; Clontech) and HA- GluD2 or its mutants using Lipofectamine 2000 (Invitrogen). The next day, HEK cells were seeded into the cerebellar culture on day in vitro (div) 12 (2×10^4 cells / cm²). Experiments were performed on div 14.

Immunocytochemistry

Cell cultures on coverslips were fixed with PBS containing 4 % paraformaldehyde and 4 % sucrose for 10 min at room temperature. After permeabilization with PBS containing 0.5 % Tween 20, samples were processed for immunofluorescent staining. After washing, coverslips were mounted with glycerol-based mounting medium AntiFade (Invitrogen). Images were captured with an FV1000 confocal laser scanning microscope (Olympus). The primary and secondary antibodies used were guinea pig anti-vglut1 antibody (Millipore; 1:20,000), Alexa 568-conjugated anti-rabbit or guinea pig IgG antibody and Alexa 488-conjugated anti-mouse IgG antibody (Invitrogen; 1:400 for each). For cell surface staining of HA- GluD2 or its mutants, cell cultures were incubated in the culture medium containing monoclonal anti-HA antibody (Roche; 5 μ g / ml) for 10 min at 37 °C. After fixation, samples were processed for immunostaining without permeabilization.

Overlay assay

For preparation of culture medium with myc-Cbln1, HEK cells were transfected with myc-Cbln1 and incubated for 2 days. HEK cells on coverslips were transfected with HA- GluD2 or its mutants using Lipofectamine™ 2000. The next day, HEK cells were incubated in the culture medium with myc-Cbln1 for 4 hours at 37 °C. Then, cells were incubated in the culture medium containing monoclonal anti-HA antibody (1 μ g / ml) and rabbit anti-myc antibody (abcam; 1 μ g / ml) for 1 hour at 37 °C. After fixation, samples were processed for immunostaining without permeabilization.

Image Analysis

For quantification of the accumulation of vglut1 on HEK cells, the cells' edges were defined using the EGFP signal by thresholding and smoothing. Then, the vglut1-immunopositive fluorescent intensity on the defined edges was measured.

For quantification of binding myc-Cbln1 on HEK cells expressing HA- GluD2 or its mutants, HA-immunopositive regions were defined by thresholding. Then, myc-immunopositive fluorescent intensity on the defined regions was measured. Images were analyzed with ImageJ (National Institutes of Health; rsb.info.nih.gov/ij/).

Statistics

All data were expressed as mean \pm SEM. One-way analysis of variance with Steel-Dwass's multiple comparison tests was used to determine the significance of differences.

Results and Discussion

GluD2-flap is essential for induction of presynaptic differentiation

Recently, the overall structure of GluA2 tetramer has been determined by X-ray crystallography [22-24]. It was the flap loop that is located on the distal end of GluA2 and closest to the presynaptic terminals. All iGluRs have the sequences corresponding to the flap loop of GluA2 (Fig 1A). The sequence similarity between the flap loop of GluD2 (GluD2-flap) and that of GluD1 was 84 %, whereas that between GluD2-flap and the flap loops of iGluRs other than GluD1 were at most 33 %. The high sequence similarity between GluD2-flap and GluD1-flap suggests that they have

related roles. In accord with this, it was reported that GluD1 also induces presynaptic differentiation [9, 25]. Thus we hypothesized that GluD2-flap is responsible for the induction of presynaptic differentiation.

We first asked if GluD2-flap is required for the induction of presynaptic differentiation. To address this issue, GluD2-flap was divided into four segments and all residues in each segment were substituted by alanine (mut_flap1-4) (Fig. 1B). HEK cells expressing GluD2 or its mutants together with EGFP were cocultured with cerebellar neurons for 2 days. Intense punctate immunoreactivity of vglut1, a marker protein of excitatory presynaptic terminals, was observed around HEK cells expressing GluD2 and EGFP but not around those expressing only EGFP, confirming that GluD2 induces presynaptic differentiation (Fig. 2). HEK cells expressing mut_flap1 or mut_flap3 did not induce accumulation of vglut1, indicating that there are essential residues in the first and third segments. In contrast, HEK cells expressing mut_flap2 or mut_flap4 induced accumulation of vglut1 comparable to those expressing GluD2. We confirmed that all mutants were expressed on the surface of HEK cells (Fig. 3A).

We then asked which residues contribute to the induction of presynaptic differentiation. To address this issue, we substituted each residue in the first and third segments of GluD2-flap by alanine one by one. HEK cells expressing the R321A or W323A mutant did not induce accumulation of vglut1 (Fig. 2), indicating that Arg321 and Trp323 in the first segments contribute to the induction of presynaptic differentiation. In contrast, single amino acid substitution in the third

segment had no significant effect on the induction of presynaptic differentiation (data not shown), suggesting that cooperative interaction among four residues in the third segment is required for the synaptogenic activity of GluD2.

Flap loop regulates binding of Cbln1 to GluD2

It was reported recently that the synaptogenic activity of GluD2 is mediated by Cbln1 [13, 14]. Cbln1 is secreted from cerebellar granule neurons and binds to GluD2-expressing cells in a manner dependent on GluD2-NTD. GluD2-expressing cells are unable to induce presynaptic differentiation against Cbln1-null cerebellar granule neurons. Therefore, we asked if GluD2-flap is responsible for the binding of Cbln1. To address this issue, the culture medium containing myc-tagged Cbln1 (myc-Cbln1) was applied to HEK cells expressing GluD2 or its mutants and myc-Cbln1 on the cell surface was observed by immunostaining without permeabilization. Intense immunoreactivity of myc-Cbln1 was observed on HEK cells expressing GluD2 but not on control cells expressing only EGFP (Fig. 3A). Significant immunoreactivity of myc-Cbln1 was detected on HEK cells expressing mut_flap2 or mut_flap4 compared with the control cells, but not on those expressing mut_flap1 or mut_flap3, indicating that the first and third segments of the flap loop are indispensable for the binding of myc-Cbln1 to GluD2 (Fig. 3). Furthermore, the immunoreactivity of myc-Cbln1 was not observed on HEK cells expressing R321A or W323A, whereas significant labeling of myc-Cbln1 was detected on those expressing K322A (Fig. 3). We also confirmed that single amino

acid substitution in the third segment interacted with Cbln1 (data not shown). The surface expression levels of mutants were not significantly different from that of native GluD2 (data not shown). These results indicate that GluD2-flap regulates the binding of Cbln1, and that especially Arg321 and Trp323 are essential residues for binding of GluD2 to Cbln1. All mutants unable to bind to Cbln1 failed to induce presynaptic differentiation, whereas the other mutants bound to Cbln1 and induced presynaptic differentiation. Thus, GluD2-flap plays crucial roles in both the induction of presynaptic differentiation and the binding of Cbln1. These results support and extend the reported finding that GluD2 induces presynaptic differentiation at PF-PN synapses through binding of its NTD to Cbln1, which further interacts with β -neurexin [13, 14].

Homology modeling of GluD2-NTD

We demonstrated that Arg321 and Trp323 in GluD2-flap are especially required for the binding of GluD2 to Cbln1. To gain insight into how these two residues in the flap loop contribute to the binding of Cbln1, we submitted the amino acid sequence of GluD2-NTD as a query to the Esypred3D web server [21] using the structure of GluA2-NTD [23] as a template, and obtained a model structure (Fig. 4A). GluD2-NTD was a clam-shell like structure consisting of two lobular domains connected by two hinge loops. When the model was superimposed on the tetrameric structure of GluA2 [24], GluD2-flap was located on the distal end of GluD2 and closest to a presynaptic varicosity as well as GluA2-flap (Fig. 4A). In this model structure, the side chains of

Arg321 and Trp323 were protruded to the outside of GluD2-NTD (Fig. 4B), suggesting that the side chains of Arg321 and Trp323 might be served for the binding interface with Cbln1.

Conclusions

GluD2 selectively expressed on the postsynaptic spine of cerebellar PN induces presynaptic differentiation through the interaction with Cbln1. Here, we showed that the flap loop in the GluD2-NTD (Arg321-Trp339) is critical for the binding of Cbln1 and for the induction of presynaptic differentiation. In particular, Arg321 and Trp323 are essential for the function.

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Fig. 1. The amino acid sequences of the flap loops. (A) Multiple alignment of the flap loops of iGluR family. Highly conserved residues among iGluRs are shown in red. Sequences corresponding to the flap loops are surrounded by yellow bands. (B) Constructed mutants. Substituted residues are indicated by A (red).

Fig. 2. Induction of presynaptic differentiation by GluD2 with amino acids substituted by alanine in the flap loop. (A) Representative images showing accumulation of vglut1 (magenta) on HEK cells (green) expressing GluD2 or its mutants together with EGFP. Scale bar: 10 μ m. (B) Vglut1 signal intensity on HEK cells. ***: $P < 0.001$ compared with EGFP. $n = 30$ for each.

Fig. 3. Overlay assay. (A) Binding of myc-Cbln1 to HEK cells expressing GluD2. EGFP signal (EGFP) or HA signal showing expression of GluD2 mutants (other than EGFP) (green) and myc signal showing Cbln1 (magenta) are shown. Scale bar: 10 μ m. (B) Intensity of myc-Cbln1 signals on the cell surface of HEK cells expressing GluD2 or its mutants. ***: $P < 0.001$ compared with EGFP. $n = 15$ for each.

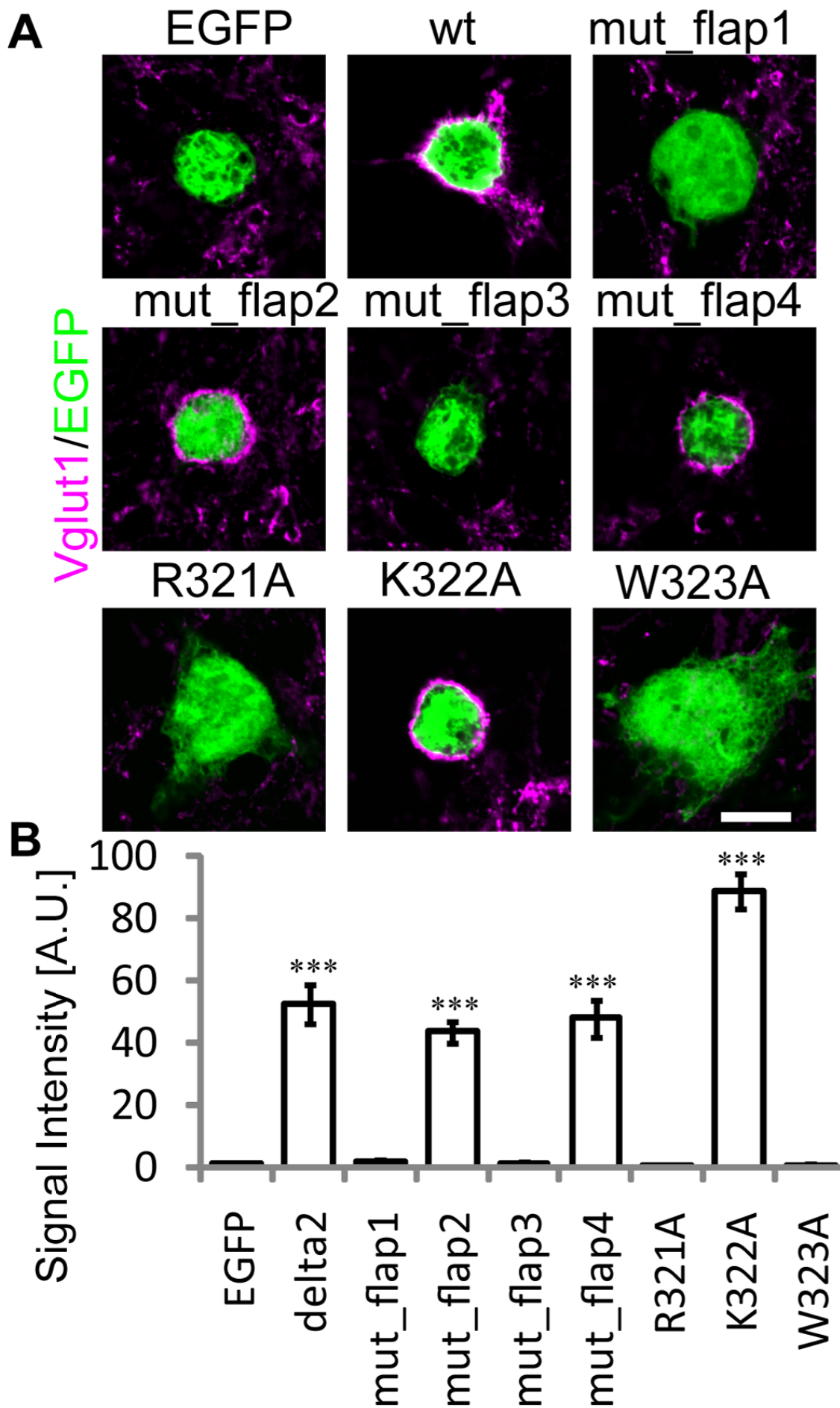
Fig. 4. Homology model of GluD2-NTD. (A) Superimposition of GluD2-NTD on the tetrameric structure of GluA2 (PDB ID: 3KG2). α -Helices (cyan), β -sheet (orange), flap loop (green) and the other loops (gray) of GluD2-NTD are shown. The four subunits in the homomer of GluA2 are shown in different pale colors. LBD, ligand binding domain; TMD, transmembrane domain. (B) An enlargement of the flap loop surrounded by the box in A is shown. Red, blue and gray balls indicate oxygen, nitrogen and carbon atoms, respectively.

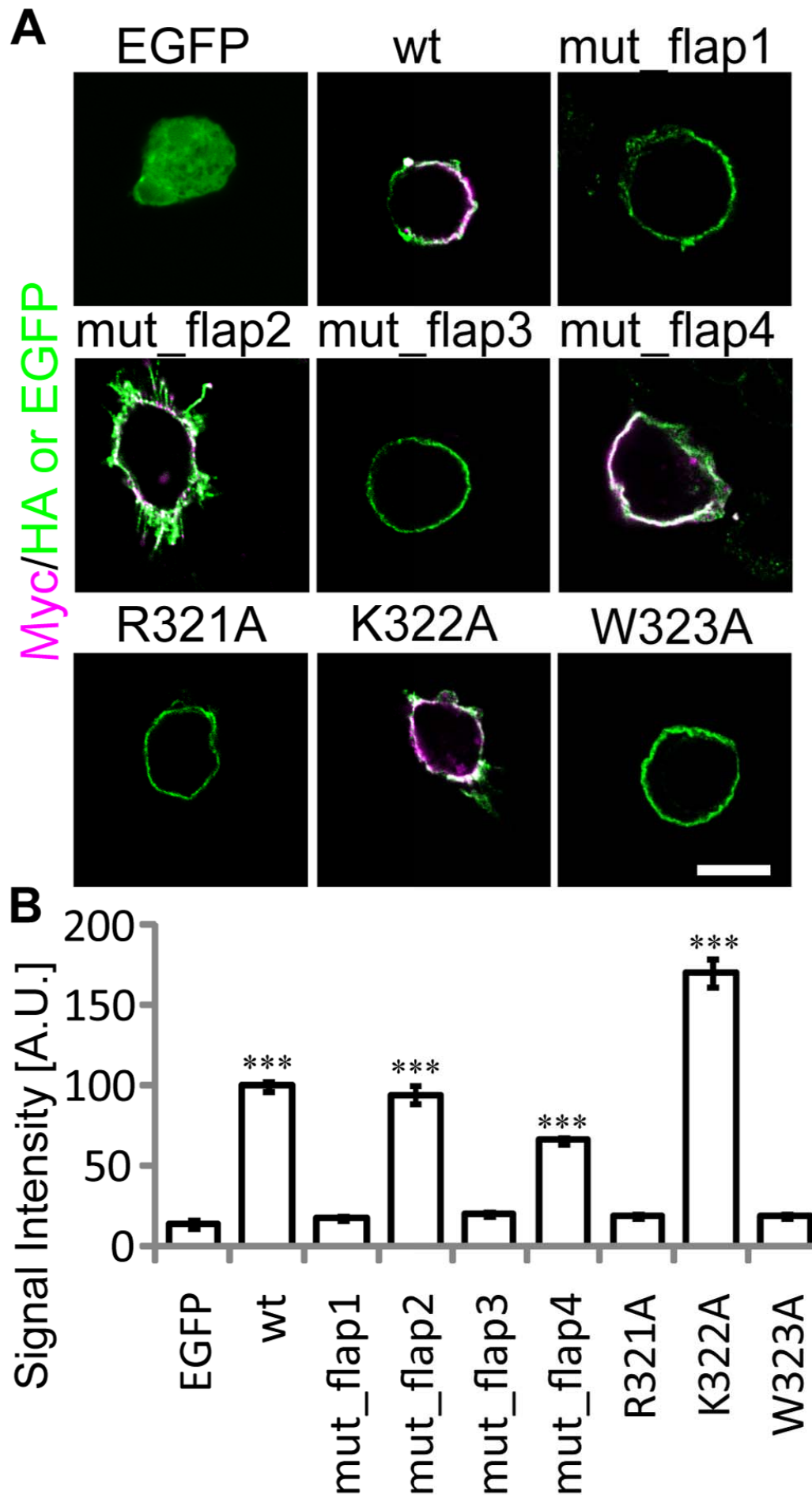
A

GluD2	L E D R K W . . H S M A S L S C I R K N S K P W
GluD1	L E D R K W . . H S M A S L N C I R K S T K P W
GluA1	R R Q R I D I S R R G N A G D C L A N P A V P W
GluA2	R K Q R I E I S R R G N A G D C L A N P A V P W
GluA3	R R Q R V D V S R R G N A G D C L A N P A V P W
GluA4	R R Q K I D I S R R G N A G D C L A N P A V P W
GluK1	S Q L T V S S L Q C H . . R H K P W
GluK2	P Q M T V S S L Q C H . . R H K P W
GluK3	P Q M T V N S L Q C H . . R H K A W
GluN1	L E K E N I T D P P R . . G . C . V G N T N I W
GluN2A	L E K F S Y I P E A K . . A S C Y G Q A E K P E
GluN2B	L S E H S F I P E P K . . S S C Y N T H E K R I

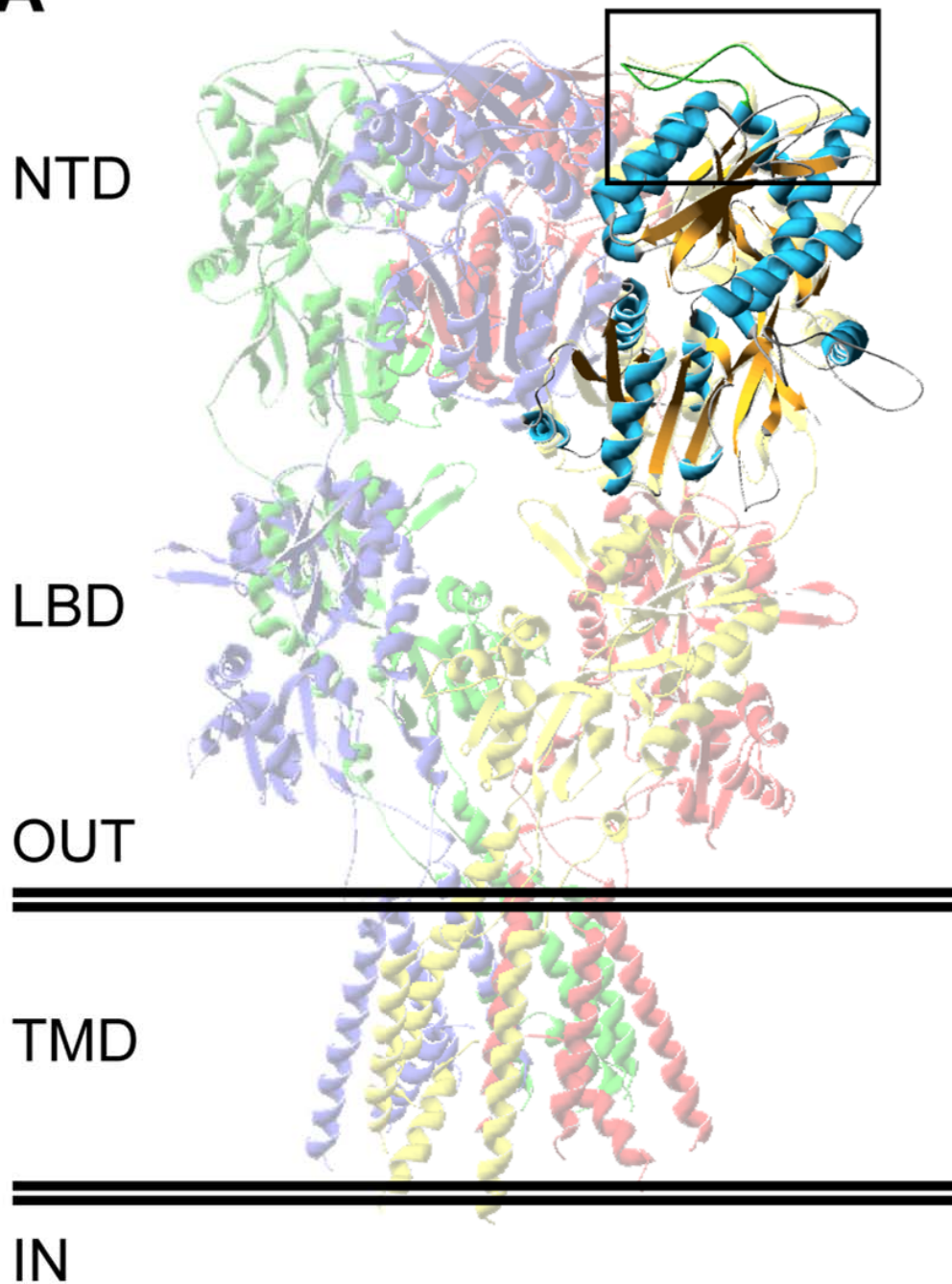
B

	321	336
wt	R K W H S M A S L S C I R K N S K	
mut_flap1	A A A H S M A S L S C I R K N S K	
mut_flap2	R K W A A A A S L S C I R K N S K	
mut_flap3	R K W H S M A A A A I R K N S K	
mut_flap4	R K W H S M A S L S C A A A A A	





A



B

